The *Saccharomyces cerevisiae* Ca^{2+} channel Cch1pMid1p is essential for tolerance to cold stress and iron toxicity

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Abstract **Cch1p and Mid1p are components of a high-affinity Ca^{2+}-permeable channel in the yeast plasma membrane. Here, we show that growth of mutants in the Cch1pMid1p channel is markedly hypersensitive to low temperature and to high iron concentration in the medium. Both phenotypes were suppressed by high Ca^{2+} concentration. Iron stress elicited an increased Ca^{2+} influx into both wild type and *cch1*Δ*mid1*Δ yeast. Inhibition of calcineurin strongly depressed growth of iron-stressed wild type yeast, indicating that calcineurin is a downstream element of the iron stress response. Iron hypersensitivity of the *cch1*Δ*mid1*Δ mutant was not associated with an increased iron uptake. An involvement of oxidative stress in the iron-hypersensitive phenotype was indicated by the findings that the antioxidants tocopheryl acetate and (ethyl)glutathione improved growth and viability of the iron-stressed mutant. Further, the degree of glutathione oxidation was increased in the presence of iron. The results indicate that iron stress leads to an increased oxidative poise and that Cch1pMid1p is essential to tolerate this condition.**

Keywords: Calcium; Channel; Cold stress; Iron toxicity; *Saccharomyces cerevisiae*

1. Introduction

A wide range of developmental and stress signals is transduced into cellular responses through elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) [1,2]. In many cases, these changes in $[Ca^{2+}]_{cyt}$ are elicited through opening of plasma membrane Ca^{2+}-permeable channels that serve to catalyze influx of Ca^{2+} down its electrochemical potential. In yeast, Ca^{2+} influx from the external medium into the cytosol has been shown to be elicited by hypertonic shock, hypotonic shock, cold stress, and salt stress [3,4]. The identity of the Ca^{2+} entry pathway has been a matter of speculation. The yeast plasma membrane contains a protein, Cch1p, with high homology to the α_1 pore-forming subunit of animal L-type voltage-gated Ca^{2+} channels [5,6]. Cch1p interacts with a second subunit, Mid1p, to catalyze high-affinity Ca^{2+} influx into yeast [7,8]. In Ca^{2+}-rich medium, deletion of either the *MID1* or the *CCH1* gene or both fails to result in an apparent phenotype. However, in selective drop out (SD) medium with low $[Ca^{2+}]$ *mid1*Δ or *cch1*Δ mutants grow less vigorously, exhibit lower Ca^{2+} influx [9], and mat **a** strains are killed by the mating pheromone α factor [5,8]. In wild type cells, exposure to α factor stimulates Ca^{2+} influx and thereby increases $[Ca^{2+}]_{cyt}$ [10]. Pheromone exposure is lethal to wild type yeast in Ca^{2+}-free media [10], suggesting that α factor-induced Ca^{2+} influx via the Cch1pMid1p Ca^{2+} channel increases $[Ca^{2+}]_{cyt}$ that in turn activates a response pathway.

Given the importance of $[Ca^{2+}]_{cyt}$ signaling in stimulus–response coupling, it is surprising that only a limited number of Ca^{2+} influx-dependent stress response pathways have been identified so far in yeast. We used *cch1*Δ and *mid1*Δ mutants defective in Ca^{2+} uptake from the external medium to search for environmental stimuli inducing a Ca^{2+}-dependent stress response in which the Cch1pMid1p channel is involved. Two new phenotypes were identified: hypersensitivity to iron and to cold stress. Whereas cold stress has been shown previously to induce a continuous rise of $[Ca^{2+}]_{cyt}$ [3], a possible involvement of Ca^{2+} signaling in iron tolerance has not been suspected hitherto.

Iron toxicity may be caused by direct ionic effects, such as interference in the transcriptional machinery [11]. Being an effective catalyst of the Fenton reaction, iron has also been demonstrated to induce the formation of the highly reactive hydroxyl ($OH^{\cdot}$) radical [12]. Here, we show that iron-induced cell death in the *cch1*Δ*mid1*Δ yeast mutant is not due to increased iron uptake, but associated with an increased oxidative poise.

2. Materials and methods

2.1. Strains, plasmids, and media

The parental *Saccharomyces cerevisiae* strain used in this study was JK9-3da (*MAT***a**, *leu2-3*, *112*, *his4*, *trp1*, *ura3-52*, *rme1*, *HMLa*). Single and double *mid1*Δ and *cch1*Δ mutants were derived from this strain by replacing the *MID1* and *CCH1* genes by a Kan-MX cassette [5]. Absence of transcript in deletion strains was confirmed by real-time RT-PCR analysis. Yeast strains were grown in synthetic drop-out (SD) media [13] supplemented with the appropriate amino acids, concentrations of which were three times those given in [13]. Media contained 20 g L^{-1} glucose unless indicated otherwise. As determined by ICP-OES, SD medium without added calcium contained 0.1 μM Ca^{2+}.

2.2. Growth and viability assays on plates and in liquid

For drop assays, yeast strains were grown overnight in SD medium containing 1 mM Ca^{2+} to a density of 10^7 cells mL^{-1}, centrifuged, resuspended to 1×10^7 or 2.5×10^7 cells mL^{-1}, and diluted 10-, 100-

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and 1000-fold. 10 μL drops were spotted onto SD media solidified with 8 g L^{-1} low-Ca^{2+} agarose (Fluka 05071) containing supplements as indicated. Plates were incubated at 30 °C unless indicated otherwise. Growth and viability in liquid culture were quantified as follows: Yeast was grown overnight in SD medium containing 1 mM Ca^{2+} to a density of 1×10^7 cells mL^{-1}. Cells were washed once with SD medium containing 0.1 mM Ca^{2+}, resuspended to 2.5×10^6 cells mL^{-1} in SD medium containing Ca^{2+}, Fe^{2+}, and supplements as indicated in Figures, and incubated shaking (160 rpm) at 30 °C. Supplementary Fe^{2+} was added after autoclaving as freshly prepared sterile-filtered 1 M $FeCl_2$ (Fluka 44939) solution. If used, 2 mg L^{-1} FK506 (Calbiochem, Nottingham, UK) was added as 1000× stock in DMSO. Controls contained solvent only. At the time points indicated, aliquots of the culture were removed and dead cells were stained with 0.5 mg L^{-1} propidium iodide [14]. Numbers of total and dead cells were counted in duplicate using a haemacytometer (Improved Neubauer).

2.3. Radiometric quantification of iron and calcium uptake

Yeast cells growing exponentially in SD medium were harvested by centrifugation, washed, and resuspended to a density of 10^8 cells mL^{-1}. After addition of $^{55}FeCl_2$ (50 kBq mL^{-1}; specific activity 185 GBq g^{-1}) or $^{45}CaCl_2$ (50 kBq mL^{-1}; specific activity 1015 GBq g^{-1}) the cells were incubated with shaking for 20 min at 20 °C. 1 mL samples were filtered through cellulose nitrate filters (Millipore HA; 0.45 μm) and washed with 30 mL of ice-cold washing medium (5 mM $FeCl_2$ or 10 mM $CaCl_2$, 300 mM sorbitol, respectively). Filters were dissolved in scintillation cocktail (Ultima Gold, Perkin–Elmer, Beaconsfield, UK) and radioactivity was determined by liquid scintillation counting. Initial experiments showed that uptake rates were linear for at least 20 min.

2.4. Quantification of oxidised and reduced glutathione in yeast cells

Yeast was grown shaking (30 °C) in SD medium (1 mM Ca^{2+}) to a cell density of 1×10^7 cells mL^{-1}. Cells were washed once with SD medium, resuspended to 2.5×10^6 cells mL^{-1} in SD medium containing Ca^{2+} and Fe^{2+} as indicated, and further incubated for 5 h. 10 mL of culture were harvested by centrifugation and total and reduced glutathione were determined by the GSSG-glutathione reductase cycling assay [15], with adaptations for yeast [16].

3. Results and discussion

3.1. Cch1pMid1p-mediated Ca^{2+} influx is essential for cold and iron stress tolerance

CCH1 and *MID1* are thought to encode subunits of a high-affinity Ca^{2+} channel located in the yeast plasma membrane [5,6,8]. To explore the role of Cch1pMid1p-mediated Ca^{2+} influx in novel stress response pathways we used strains devoid of these proteins. Cold stress induces an increase of $[Ca^{2+}]_{cyt}$ in yeast [3]. We suspected that this Ca^{2+} influx is essential and examined growth of *cch1*Δ and *mid1*Δ single and *cch1*Δ*mid1*Δ double mutants at low temperature (Fig. 1A). At 30 °C, growth of the mutants was comparable with that of the wild type. In contrast to the wild type, growth of the mutants on low [Ca^{2+}] solid SD media was severely impaired at 10 °C. The cold-sensitivity of the *cch1*Δ*mid1*Δ mutant also manifested itself in higher doubling times and increased cell death of liquid cultures grown at 10 °C (Fig. 1B). The notion that Cch1p and Mid1p are subunits of a single channel [7] was supported by the fact that the *cch1*Δ*mid1*Δ double mutant was not more sensitive to the imposed stress than the single mutants. However, the *cch1*Δ mutant appeared to be slightly more cold-sensitive than the *mid1*Δ and *cch1mid1*Δ mutants. This was confirmed with liquid cultures grown at 10 °C, where the doubling time of *cch1*Δ (81.5 ± 5.0 h) was higher than that of *mid1*Δ (49 ± 0.8 h) and *cch1*Δ*mid1*Δ (49 ± 2.2 h). The sub-

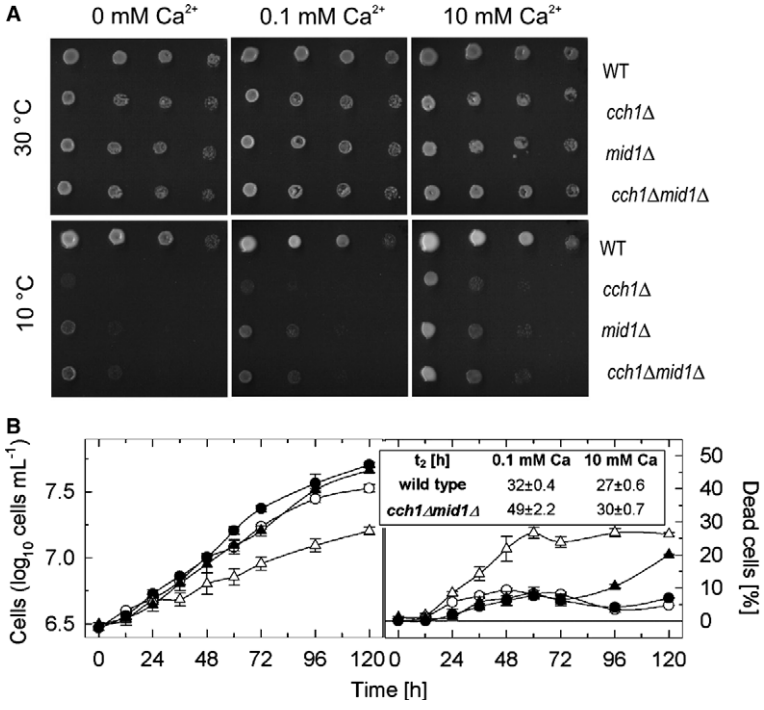

Fig. 1. Cch1p and Mid1p are required for growth at low temperature. (A) Plate assay. Yeast was spotted onto SD agarose plates containing 0 (no added), 0.1, or 10 mM $CaCl_2$. Plates were incubated at 30 and 10 °C, and scanned after 3 days and 17 days, respectively. (B) Liquid growth assay. Growth (left) and viability (right) of wild type (●, ○) and *cch1*Δ*mid1*Δ mutant (▲, △) yeast in SD medium containing 0.1 mM Ca^{2+} (○, △) or 10 mM Ca^{2+} (●, ▲). Inserted table shows doubling times calculated by linear regression of individual growth curves. Values are the means ± S.E.M. of three experiments.

unit-specific cold sensitivity might suggest that in the absence of Cch1p, expression of Mid1p can exert a negative effect, perhaps through interaction with another protein.

The cold-sensitive phenotype was partially suppressed by addition of 10 mM Ca^{2+} (Fig. 1A and B). This can be explained by the presence of an alternative, so far unidentified, Ca^{2+} influx pathway which has a lower Ca^{2+} affinity than Cch1pMid1p [9]. Although high external Ca^{2+} concentration restored growth of the mutant, it was not able to prevent cell death during prolonged periods of cold stress (Fig. 1B).

In a search for further stress response pathways requiring Ca^{2+} influx through the Cch1pMid1p channel we exposed *cch1*Δ, *mid1*Δ, and *cch1*Δ*mid1*Δ mutants to cation stress. For most divalent ions tested (Mg^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}) the sensitivities of mutants and wild type did not differ (data not shown). However, exposure to 10 mM Fe^{2+} on solid SD media containing 0.1 mM Ca^{2+} abolished growth of the mutants almost completely, but did not affect the wild type (Fig. 2A). As with the cold stress phenotype, the *cch1*Δ*mid1*Δ double mutant was not more sensitive to iron stress than the single mutants and addition of 10 mM Ca^{2+} almost completely abolished the iron hypersensitivity.

To examine the effect of iron stress on cell death, yeast was grown in liquid SD media (Fig. 2B). Under these conditions, the *cch1*Δ*mid1*Δ mutant stopped growing after 7.5 h. As demonstrated previously [5], cell death of the *cch1*Δ*mid1*Δ mutant was increased by low [Ca^{2+}] in the medium. Iron stress caused a further increase in mortality (Fig. 2B). Both, growth arrest and cell death were alleviated by supplementing the medium with 10 mM Ca^{2+}. To determine whether there are interactions between the cold and iron stress responses, we tested whether exposure to cold stress affected the tolerance to iron stress and vice versa. At 10 °C, wild type growth was severely compromised by the presence of 1 mM Fe^{2+} and growth of the mutants was completely abolished at this concentration (data not shown). Rescue of the mutant phenotype by 10 mM Ca^{2+} was only partial, demonstrating that cold and iron stress act additively with respect to Ca^{2+} dependence for stress survival.

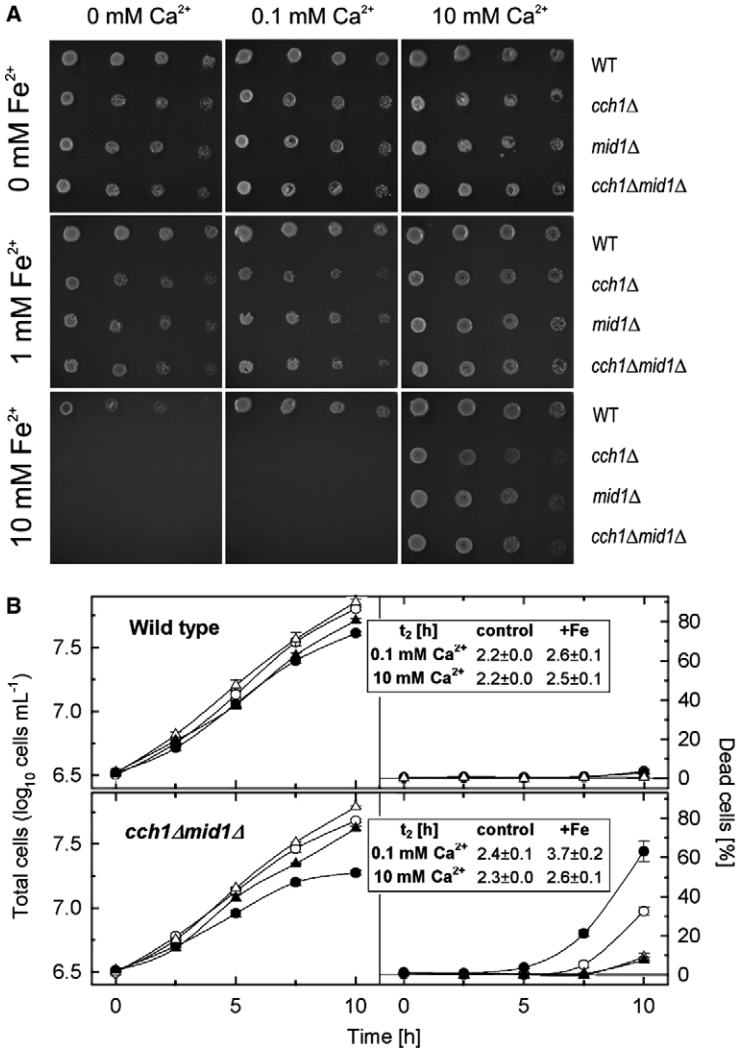

Fig. 2. Cch1p and Mid1p are required for growth at high iron concentration. (A) Plate assay. Yeast was spotted onto SD agarose plates containing 0 (no added), 0.1, or 10 mM $CaCl_2$ and 0, 1, or 10 mM supplementary Fe^{2+}. Plates were incubated at 30 °C and scanned after 3 days. (B) Liquid growth assay. Growth (left) and viability (right) of wild type (upper panel) and *cch1*Δ*mid1*Δ mutant (lower panel) yeast in SD medium containing 0.1 mM Ca^{2+} (●, ○) or 10 mM Ca^{2+} (▲, △) and supplemented with no (○, △) or 10 mM Fe^{2+} (●, ▲). Inserted tables show doubling times calculated by linear regression of individual growth curves. Values are the means ± S.E.M. of five experiments.

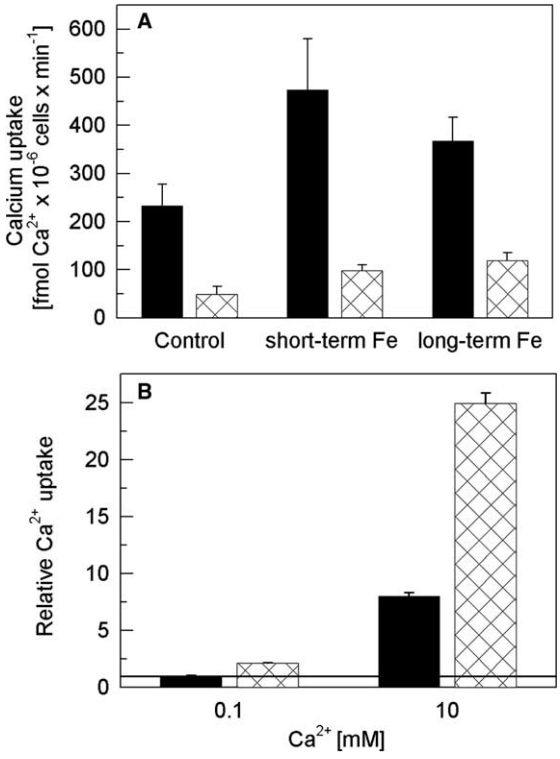

Fig. 3. Influx of $^{45}Ca^{2+}$ is increased during iron stress. (A) 1 mM Fe^{2+} was either included in growth and uptake media (long-term Fe) or added immediately prior to addition of the radioisotope (short-term Fe). Black bars: wild type, hatched bars: *cch1*Δ*mid1*Δ mutant. Values are the means ± S.E.M. of six experiments. (B) The *cch1*Δ*mid1*Δ mutant was grown for 3 h in medium containing 0.1 or 10 mM Ca^{2+} and supplemented with no (black bars) or 1 mM Fe^{2+} (hatched bars) before uptake of $^{45}Ca^{2+}$ from the same media was determined. Values are the means ± S.E.M. of four experiments.

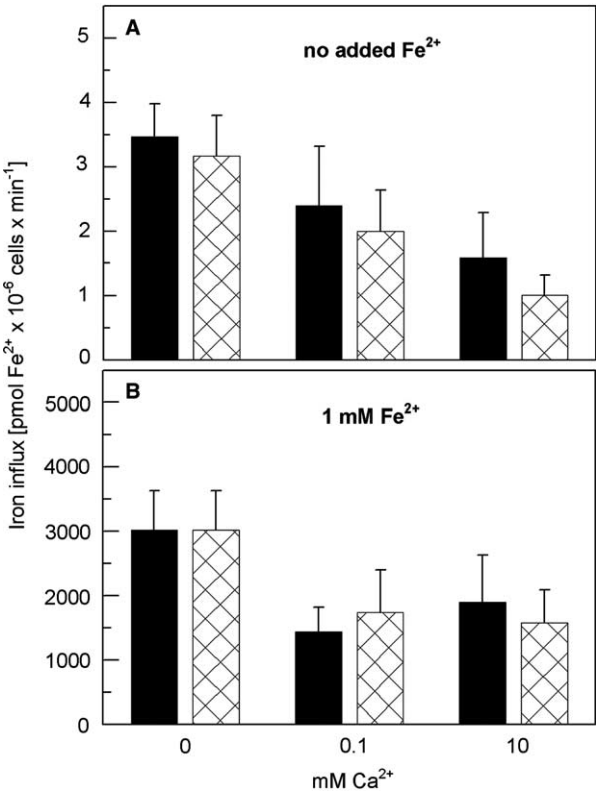

Fig. 5. Iron uptake is unaltered in the *cch1*Δ*mid1*Δ mutant. ^{55}Fe uptake by exponentially growing cultures of wild type (black bars) and *cch1*Δ*mid1*Δ mutant (crossed bars) from SD medium containing Ca^{2+} as indicated and no (A) or 1 mM (B) supplementary Fe^{2+}. Values are the means ± S.E.M. of seven experiments.

The nature of the involvement of the Cch1pMid1p channel in facilitating growth at potentially toxic concentrations of iron was further investigated. One possibility is that iron stress induces an enhanced Ca^{2+} influx which is involved in iron stress signaling. We tested this possibility by radiometric monitoring of Ca^{2+} influx in response to iron. In accord with previous studies [5–7], Ca^{2+} uptake was substantially decreased in the *cch1*Δ*mid1*Δ mutant (Fig. 3A). Ca^{2+} uptake was stimulated by a factor of about two by including Fe^{2+} in the culture medium, and also by adding Fe^{2+} at the start of the uptake exper-

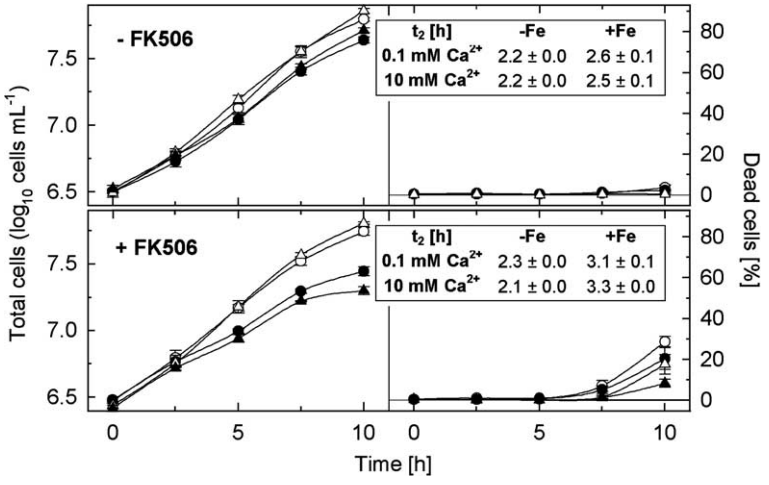

Fig. 4. Inhibition of calcineurin by FK-506 affects iron-stressed wild type yeast. Growth (left) and viability (right) of liquid yeast cultures in SD medium containing 0.1 mM Ca^{2+} (●, ○) or 10 mM Ca^{2+} (▲, △) and supplemented with no (○, △) or 10 mM Fe^{2+} (●, ▲) and no (upper panel) or 2 μg mL^{-1} FK506 (lower panel). Inserted tables show doubling times calculated by linear regression of individual growth curves. Values are the means ± S.E.M. of four independent experiments.

iment (Fig. 3A). Interestingly, iron stress also increased the residual Ca^{2+} uptake in the *cch1Δmid1Δ* mutant. Hence both Cch1pMid1p and an alternative pathway are activated by iron. The rapidity of the response to iron suggests that its effects on Ca^{2+} uptake are manifested, at least in part, post-translationally.

Ca^{2+} uptake by the *cch1Δmid1Δ* mutant was eight-fold increased by raising the $[Ca^{2+}]$ in the medium from 0.1 to 10 mM (Fig. 3B). The increased Ca^{2+} uptake from high $[Ca^{2+}]$ medium was further stimulated by iron stress. This supports the notion that enhanced Ca^{2+} influx compensates for the reduced Ca^{2+} uptake capacity in the mutant.

The observation that Ca^{2+} influx through Cch1pMid1p protects yeast cells against iron toxicity strongly suggests the presence of a Ca^{2+}-dependent iron stress response pathway. The Ca^{2+}- and calmodulin-dependent protein phosphatase calcineurin has been shown in yeast to be a common link in a number of Ca^{2+}-based signaling pathways [4,17] and also to affect cytosolic Ca^{2+} homeostasis by feedback-regulation of Ca^{2+} transporters [9,18]. Accordingly, growth of FK506-inhibited cells was found to be hypersensitive to iron stress (Fig. 4). High $[Ca^{2+}]$ did not increase the growth of the iron-stressed FK506-inhibited cells. In contrast to growth, cell death of FK506-inhibited cells was not exacerbated by iron stress. Addition of 10 mM Ca^{2+} alleviated FK506-induced cell death of both iron-stressed and unstressed cells. These results suggest that calcineurin activation is necessary to maintain growth under iron stress, whereas mechanisms to prevent iron-induced cell death are calcineurin-independent.

3.2. The nature of iron hypersensitivity of the *cch1Δmid1Δ* mutant

A range of possible mechanisms for the iron hypersensitivity of the *cch1Δmid1Δ* mutant is conceivable. As in the *AFT1-1*^{up} mutant [11], deregulation of iron uptake systems may have caused iron overload. However, radiometric flux studies with $^{55}Fe^{2+}$ showed that iron influx was similar in wild type and mutant, both at low iron concentration and from medium supplemented with 1 mM Fe^{2+} (Fig. 5). Interestingly, in both mutant and wild type, iron influx was decreased by raising $[Ca^{2+}]$ to only 0.1 mM. It remains to be examined whether this is due to a direct effect of Ca^{2+} on the low-affinity iron transport system.

We suspected that intracellular iron metabolism or compartmentation may be disrupted in the *cch1Δmid1Δ* mutant. Iron is a redox-active transition metal and catalyzes the formation of highly reactive hydroxyl radicals from H_2O_2 in the Fenton reaction [12]. Mutants in several genes whose products are involved in cellular iron homeostasis (e.g., Aft1p and Aft2p [19], Yhf1p [20], Sod1p [21,22]) are therefore hypersensitive to oxi-

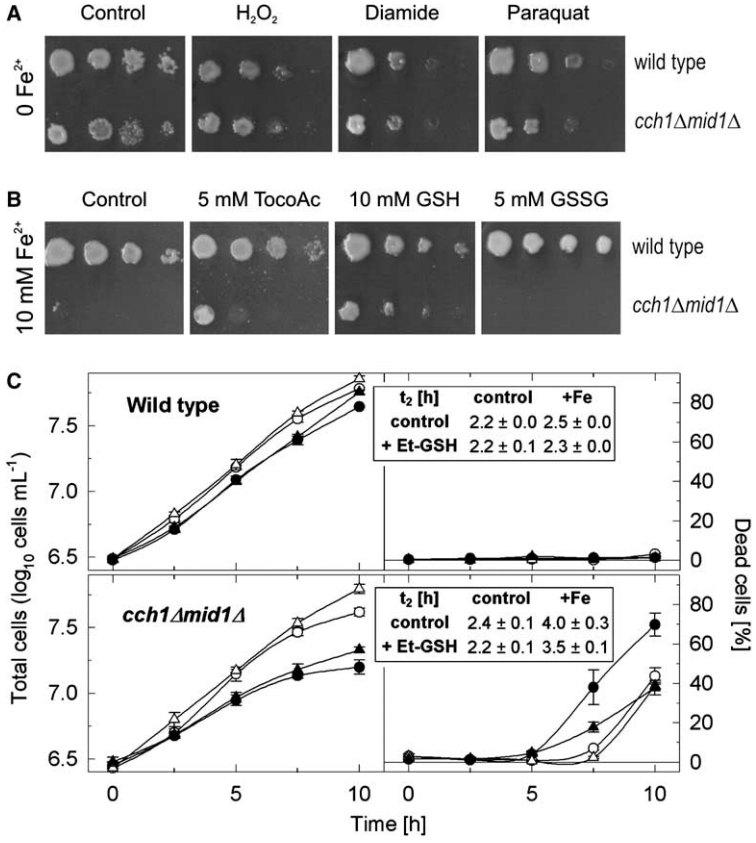

Fig. 6. The involvement of oxidative stress in the iron-hypersensitive phenotype. (A) The *cch1Δmid1Δ* mutant is not hypersensitive to various oxidants. Wild type and *cch1Δmid1Δ* yeast was spotted onto SD agarose plates containing 0.1 mM Ca^{2+}, and H_2O_2 (2 mM), diamide (0.75 mM), paraquat (0.5 mM), or no oxidant. (B, C) The iron-hypersensitive phenotype of the *cch1Δmid1Δ* mutant is alleviated by antioxidants. (B) Plate assay. Yeast was spotted onto SD agarose plates containing 0.1 mM Ca^{2+}, 10 mM Fe^{2+}, and tocopheryl acetate (5 mM), reduced glutathione (10 mM), oxidised glutathione (5 mM) or no supplement. (C) Liquid growth assay. Yeast was grown in SD medium containing 0.1 mM Ca^{2+} and supplemented with no (○, △) or 10 mM Fe^{2+} (●, ▲) and no (●, ○) or 10 mM ethyl glutathione (▲, △). Inserted tables show doubling times calculated by linear regression of individual growth curves. Values are the means ± S.E.M. of five experiments.

dising agents. However, the mutant was not hypersensitive to externally applied H_2O_2, and sensitivities of wild type and mutant towards the thiol oxidant diamide [23] and the superoxide generator paraquat [22] were comparable (Fig. 6A). Other redox-active transition metals, such as Cu^{2+}, have also been implicated in oxidative stress [12,24]. On SD (0.1 mM Ca^{2+}) plates, growth of both wild type and *cch1Δmid1Δ* mutant was partially depressed by 0.5 mM Cu^{2+}, but an increased sensitivity of the mutant was not observed (data not shown). It is possible that toxicity of Cu^{2+} per se is apparent at concentrations lower than those at which redox effects would occur.

The results indicate that the iron-hypersensitive phenotype of the *cch1Δmid1Δ* mutant is not caused by a general hypersensitivity to oxidative stress. However, because the half-life of the hydroxyl radical is extremely short, iron-induced oxidative damage is highly localised and dependent on the location of the catalytic iron [12]. This idea is supported by the fact that iron sensitivity of the *cch1Δmid1Δ* mutant was alleviated by the antioxidant tocopheryl acetate and more so by reduced glutathione (GSH) (Fig. 6B), which is the main antioxidant in yeast [25–27]. The beneficial effect of the lipophilic tocopheryl acetate indicates that iron hypersensitivity might be associated with membrane damage. Iron-induced cell death of the mutant in liquid culture was also strongly decreased by ethyl glutathione (EtGSH) (Fig. 6C), a highly membrane-permeable glutathione derivative [28]. The degree of glutathione oxidation is considered to be a parameter of the intracellular redox status [25]. In both wild type and *cch1Δmid1Δ* mutant we found this parameter to be increased by exposure of the yeast to iron stress (Fig. 7), indicating that iron stress leads to an increased oxidative poise in both strains. The direct link between Ca^{2+} influx and sensitivity to iron-induced oxidative stress remains to be established.

3.3. A general significance of Ca^{2+} in iron toxicity?

In certain conditions, all higher organisms can encounter potentially toxic concentrations of iron. Plants growing on waterlogged soils frequently show symptoms of iron toxicity because the availability of iron is strongly increased under the reducing conditions that prevail in those soils [29]. Iron overload also plays an important role in a wide range of human disorders, and has been shown to cause or exacerbate neurodegenerative diseases [30], cardiopathies [31], and ischemic tissue injuries [32]. Iron has been shown to induce Ca^{2+} influx and raise $[Ca^{2+}]_{cyt}$ in mammalian cells [33–35], but a requirement of Ca^{2+} influx for survival of an increased iron load has not been suggested before. It is tempting to speculate that enhanced Ca^{2+} influx may represent a conserved element in iron signaling pathways.

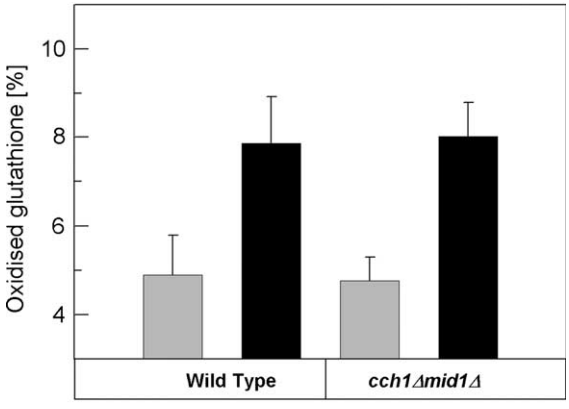

Fig. 7. Iron stress increases glutathione oxidation in wild type and *cch1Δmid1Δ* mutant. Yeast was grown in SD medium (0.1 mM Ca^{2+}) supplemented with 0 (grey bars) or 10 mM Fe^{2+} (black bars). Total and oxidised glutathione were determined after 5 h of treatment. Values are the means ± S.E.M. of three experiments.

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References

[1] Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. Plant Cell 14, S401–S417.
[2] Clapham, D.E. (1995) Calcium signaling. Cell 80, 259–268.
[3] Batiza, A.F., Schulz, T. and Masson, P.H. (1996) Yeast respond to hypotonic shock with a calcium pulse. J. Biol. Chem. 271, 23357–23362.
[4] Matsumoto, T.K., Ellsmore, A.J., Cessna, S.G., Low, P.S., Pardo, J.M., Bressnan, R.A. and Hasegawa, P.M. (2002) An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of *Saccharomyces cerevisiae*. J. Biol. Chem. 277, 33075–33080.
[5] Fischer, M., Schnell, N., Chattaway, J., Davies, P., Dixon, G. and Sanders, D. (1997) The *Saccharomyces cerevisiae* CCH1 gene is involved in calcium influx and mating. FEBS Lett. 419, 259–262.
[6] Paidhungat, M. and Garret, S. (1997) A homolog of mammalian, voltage-gated calcium channels mediates yeast pheromone-stimulated Ca^{2+} uptake and exacerbates the *cdc1* (Ts) growth defect. Mol. Cell. Biol. 17, 6339–6347.
[7] Locke, E.G., Bonilla, M., Liang, L., Takita, Y. and Cunningham, K.W. (2000) A homolog of voltage-gated Ca^{2+} channels stimulated by depletion of secretory Ca^{2+} in yeast. Mol. Cell. Biol. 20, 6686–6694.
[8] Iida, H., Nakamura, H., Ono, T., Okumara, M.S. and Anraku, Y. (1994) *MID1*, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca^{2+} influx and mating. Mol. Cell. Biol. 14, 8259–8271.
[9] Muller, E.M., Locke, E.G. and Cunningham, K.W. (2001) Differential regulation of two Ca^{2+} influx systems by pheromone signaling in *Saccharomyces cerevisiae*. Genetics 159, 1527–1538.
[10] Iida, H., Yagawa, Y. and Anraku, Y. (1990) Essential role for induced Ca^{2+} influx followed by $[Ca^{2+}]$i rise in maintaining viability of yeast cells late in the mating pheromone response pathway. J. Biol. Chem. 265, 13391–13399.
[11] Philpott, C.C., Rashford, J., Yamaguchi-Iwai, Y., Rouault, T.A., Dancis, A. and Klausner, R.D. (1998) Cell cycle arrest and inhibition of G_1 cyclin translation by iron in AFT1-1^{up} yeast. EMBO J. 17, 5026–5036.
[12] Halliwell, B. and Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Meth. Enzymol. 186, 1–85.
[13] Sherman, F. (2002) Getting started with yeast. Meth. Enzymol. 350, 3–41.
[14] Peiter, E., Imani, J., Yan, F. and Schubert, S. (2003) A novel procedure for gentle isolation and separation of intact infected and uninfected protoplasts from the central tissue of *Vicia faba* L. root nodules. Plant Cell Environ. 26, 1117–1126.
[15] Anderson, M.E. (1985) Determination of glutathione and glutathione disulfide in biological samples. Meth. Enzymol. 113, 548–555.
[16] Cuozzo, J.W. and Meister, C.A. (1999) Competition between glutathione and protein thiols for disulfide-bond formation. Nature Cell Biol. 1, 130–135.
[17] Bonilla, M., Nastase, K.K. and Cunningham, K.W. (2002) Essential role of calcineurin in response to endoplasmic reticulum stress. EMBO J. 21, 2343–2353.

[18] Cunningham, K.W. and Fink, G.R. (1996) Calcineurin inhibits *VCX1*-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 16, 2226–2237.
[19] Blaiseau, P.-L., Lesuisse, E. and Camadro, J.-M. (2001) Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. J. Biol. Chem. 276, 34221–34226.
[20] Babcock, M., De Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M. and Kaplan, J. (1997) Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. Science 276, 1709–1712.
[21] De Freitas, J.M., Liba, A., Meneghini, R., Selverstone Valentine, J. and Butler Gralla, E. (2000) Yeast lacking Cu–Zn superoxide dismutase show altered iron homeostasis. Role of oxidative stress in iron metabolism. J. Biol. Chem. 275, 11645–11649.
[22] Srinivasan, C., Liba, A., Imlay, J.A., Selverstone Valentine, J. and Butler Gralla, E. (2000) Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. J. Biol. Chem. 275, 29187–29192.
[23] Wemmie, J.A., Steggerda, S.M. and Moye-Rowley, W.S. (1997) The *Saccharomyces cerevisiae* AP-1 protein discriminates between oxidative stress elicited by the oxidants H_2O_2 and diamide. J. Biol. Chem. 272, 7908–7914.
[24] Avery, S.V. (2001) Metal toxicity in yeasts and the role of oxidative stress. Adv. Appl. Microbiol. 49, 111–142.
[25] Jamieson, D.J. (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14, 1511–1527.
[26] Grant, C.M. (2001) Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol. Microbiol. 39, 533–541.
[27] Stephen, D.W.S. and Jamieson, D.J. (1996) Glutathione is an important antioxidant molecule in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 141, 207–212.
[28] Anderson, M.E., Levy, E.J. and Meister, A. (1994) Preparation and use of glutathione monoesters. Meth. Enzymol. 234, 492–499.
[29] Sahrawat, K.L. (2004) Iron toxicity in wetland rice and the role of other nutrients. J. Plant Nutr. 27, 1417–1504.
[30] Thomas, M. and Jankovic, J. (2004) Neurodegenerative disease and iron storage in the brain. Curr. Opin. Neurol. 17, 437–442.
[31] Limdi, J.K. and Crampton, J.R. (2004) Hereditary haemochromatosis. QJM Int. J. Med. 97, 315–324.
[32] Selim, M.H. and Ratan, R.R. (2004) The role of iron neurotoxicity in ischemic stroke. Ageing Res. Rev. 3, 345–353.
[33] Stoyanovsky, D.A. and Cederbaum, A.I. (1998) Redox-cycling of iron ions triggers calcium release from liver microsomes. Free Rad. Biol. Med. 24, 745–753.
[34] Anghileri, L.J. and Thouvenot, P. (1998) ATP in iron overload-induced intracellular calcium changes. Int. J. Mol. Med. 1, 869–873.
[35] Cheng, Y., Wixom, P., James-Kracke, M.R. and Sun, A.Y. (1994) Effects of extracellular ATP on Fe^{2+}-induced cytotoxicity in PC-12 cells. J. Neurochem. 63, 895–902.